Monoclonal Antibody 44-3A6 as a Probe for a Novel Antigen Found on Human Lung Carcinomas with Glandular Differentiation

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ABSTRACT

This paper describes an immunoglobulin G1 mouse monoclonal antibody (MCA) 44-3A6 directed against a human adenocarcinoma of the lung, cell line A549. This hybrid is a fusion product of the mouse myeloma SP 2/0.Ag14 and spleen cells from a BALB/c mouse which had been hyperimmunized with A549. Live cell radioimmunoassays, immunofluorescences, and fluorescent activated cell sorter analysis indicate that MCA 44-3A6 reacts with a cell surface antigen. Western blot analysis identifies a major antigen band with the apparent molecular weight of 40,000. Enzyme treatment of A549 target plates shows that the antigen is sensitive to proteases. This MCA does not react with carcinomembryonic antigen. Patients having a variety of different carcinomas of the lung demonstrated binding to be restricted to tumors with features of "glandular" differentiation. This MCA may have clinical and shows a cell surface and/or cytoplasmic staining pattern. This paper describes one such MCA which distinguishes lung carcinomas with "glandular" differentiation from the other lung carcinomas lacking these features, and it may be useful as both a diagnostic and therapeutic agent.

MATERIALS AND METHODS

Cell Lines. Well-characterized human lung carcinoma cell lines were grown as previously described (2-4).

Production of Monoclonal Antibody 44-3A6. The antibody was produced using classic hybridoma technology (5). A BALB/c mouse was hyperimmunized with a well-characterized human adenocarcinoma cell line, A549, and its splenocytes were fused to the nonsecreting, mutant mouse myeloma cell line, SP 2/0.Ag14, as previously described. Polyethylene Glycol 1450 (Fisher Scientific Company, Chicago, IL) was used as the fusion agent. Only one hybrid line was derived from any one of the original 480 wells in plating the hybrids after the fusion. The hybridoma producing monoclonal antibody 44-3A6 was selected after initial screening and was subsequently cloned 3 times on soft agar (6). Ouchterlony analysis was performed on supernatant which had been concentrated 25 times.

Ascites fluid was prepared by i.p. implantation of 1 x 107 hybridoma cells in BALB/c mice which had been pristane primed 3-7 days prior to injection. Monoclonal antibody 44-3A6 was purified by 50% ammonium sulfate precipitation, followed by affinity chromatography using Staphylococcus Protein A (7, 8). Protein concentrations were determined using the Bio-Rad protein assay kit using bovine plasma γ-globulin as a standard. Supernatants, ascites fluid, and purified antibody samples were stored with a final concentration of 0.02% sodium azide or sterile filtered, at either 4°C or -70°C.

Radioimmunoassays. Human lung carcinoma cell line lysates were made essentially as previously reported (4). Supernatants, ascites fluid, or purified antibody (50 μl of each) was incubated at room temperature for 1 h in 95.5-well polyvinyl chloride target plates of each cell line tested. After being washed 7 times with 250-μl volumes of PBS, the target plates were incubated with 5 x 104 cpm of 125I-sheep anti-mouse F(ab')2 fragments (Amersham, Arlington Heights, IL) in a 50-μl volume. The plates were washed 7 times as stated above after a 1-h incubation, and they were counted in a gamma counter for 1 min. Controls included (when appropriate) no primary antibody, SP 2/0.Ag14 supernatant, normal mouse serum, and a monoclonal antibody at the same concentration and identical isotype. Binding ratios were calculated as previously described (4).

Enzyme-treated Radioimmunoassays. Target plates of human lung carcinoma cell line lysates were made as described above, and each well was treated with 50 μl of treatment solution. The following treatments and final concentrations were: trypsin (15.4 F units); pepsin (14 units); lipase (50 units); chymotrypsin (0.225 unit); H5IO6 (1%); β-galactosidase (5 units); neuraminidase (0.1 unit); and a-mannosidase (0.18 unit). After 2 h of treatment at optimum conditions for each treatment, the plates were washed 3 times and reblocked with 1% BSA-PBS with azide. The treated plates were then tested as described above.

Live Cell Radioimmunoassay. Human lung carcinoma cell lines were harvested at mid-log growth phase using a rubber policeman to dislodge cell lines which adhere to the flasks. Cell suspensions were triturated gently and washed 3 times with PBS. Cells (1 x 106) were resuspended...
in 100 µl of PBS containing 0.02% sodium azide and plated into microtiter wells (Immulon 2 Removewell Strips; Dynatech Laboratories, Alexandria, VA). The microtiter plates had been pretreated for 24 h with BSA (1 mg/ml) in PBS with azide. Fifty µl of supernatant or ascites fluid (diluted 1:100) were added to each well. Plates were incubated at room temperature for 30 min on a platform shaker set at low speed. After the incubation period, the cells were washed 7 times with cold PBS containing azide by centrifuging the plates at 1,000 rpm for 5 min, aspirating the supernatants, and resuspending the cells in 200 µl of PBS. 125I-sheep anti-mouse antibody F(ab')2 (50,000 cpm/µl) was added to each well after being washed and incubated as before. Each well was again washed 7 times as before, and the wells were counted in a gamma counter for 1-min intervals.

Immuno-Fluorescence. Cell lines were harvested and washed as described above. Cells (1 x 10^6/well) were spotted onto 10-well microscope slides (Celite-Link Inc., Newfield, NJ), allowed to adhere for 5 min, and air dried. The slides were either fixed for 12 min in 1% glutaraldehyde or in acetone at 0°C. Slides were stored in PBS at 4°C until used. Each well was incubated with 100 µl of supernatant for 1 h in a humidified 37°C chamber. After 3 washes with PBS, the slides were incubated with fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Cappel, Malvern, PA), washed 3 times, and viewed with a fluorescent microscope. Samples were scored on a scale of zero to plus four using a double blinded reviewer.

Fluorescent Activated Cell Sorter Analysis. Human lung carcinoma cell lines were harvested and washed as described above, adjusted to 1 x 10^6 cells/ml and aliquoted into 12 x 75 mm test tubes. After centrifuging at 1000 rpm for 5 min at 4°C, the pellet was resuspended in 100 µl of supernatant and kept on ice for 1 h. Samples were washed 3 times, and the pellet was resuspended in 100 µl of fluorescein isothiocyanate-conjugated goat anti-mouse antibody. The cells were washed 3 times after a 1-h incubation on ice and fixed in 0.5% paraformaldehyde-PBS for 10 min. Samples were stored for no longer than 2 wk in the dark at 4°C in PBS:azide. Analysis of samples was performed using a Coulter Epics V fluorescent activated cell sorter.

Immunoprecipitation and Western Blot Analysis. Radiolabeled cell line lysates were produced by growing human lung carcinoma cell lines (2 x 10^6 cells/ml) in 5% dialyzed fetal calf serum with [35S]methionine (30 µCi/ml) and L-[3-14C]-amino acids (10 µCi/ml) (Amersham, Arlington Heights, IL). After they had grown for 24 h, they were washed 3 times, resuspended in a minimal volume of solubilization buffer, and sonicated on ice. Supernatants of MCA 44-3A6 and purified antibody were then incubated with the cell lysates overnight at 4°C. After addition of rabbit anti-mouse IgG, the immune complexes were precipitated with Staphylococcus Protein A, further indicating that it belonged to an IgG subclass. Stability of this clone has been demonstrated by repeated cloning in culture with high titer antibody production. To further show that this clone was producing an antibody, 25-fold concentrated culture supernatants. Immunoperoxidase staining using the avidin:biotin:horseradish peroxidase complex method (Vector Laboratories, Burlingame, CA) was used as suggested by the manufacturer and counterstained with hematoxylin. Positive tissue was tested with each set of slides, and controls included no primary antibody or an antibody of the same isotype but different binding characteristics.

RESULTS

Screening for Selectivity of Antibody Binding. Ouchterlony analysis of 25-fold concentrated supernatant revealed that 44-3A6 had an IgG1 isotype. This MCA also bound to Staphylococcus Protein A, further indicating that it belonged to an IgG subclass. Stability of this clone has been demonstrated by repeated cloning in culture with high titer antibody production. To further show that this clone was producing an antibody, 25-fold concentrated supernatant was diluted serially and used in an RIA on positive and negative lung cancer cell lines. Shown in Chart 1 is the binding of 44-3A6 to A549 (AC) and NCI-N464 (SCLC). These data show that the antibody can be diluted 1:390,625 on A549 cell line lysate target plates and still have greater binding than at any dilution on the SCLC line NCI-N464. Similar results could be demonstrated on another positive AC cell line and several negative SCLC cell lines (data not shown). The binding characteristics of a control antibody with an IgG1 isotype showed no binding to either A549 or NCI-N464 (data not shown). Initial screening using ten human lung cell lines showed binding to AC cell lines and not to eight other lung cancer cell lines representing a variety of histological types. Table 1 shows both the cpm and binding ratios (total cpm/control cpm) to the original panel of human lung lines used to screen this MCA. Due to this unique reactivity pattern, we wished to further investigate the potential usefulness of this MCA.

Live Cell Assays for Binding to Lung Cancer Cell Lines.
Chart 1. Titration curve of MCA 44-3A6 using an RIA as described in "Materials and Methods." The positive control target cell line was A549 (adenocarcinoma) and the negative control target cell line was NCI-N464 (small cell).

Table 1

<table>
<thead>
<tr>
<th>Lysate target</th>
<th>cpm</th>
<th>Binding ratio</th>
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<tr>
<td>AC lines</td>
<td></td>
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<tr>
<td>NCI-H234</td>
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<tr>
<td>NCI-SKLU-1</td>
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<td>10.0</td>
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<td>A549</td>
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<td>11.6</td>
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<td>A427</td>
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<tr>
<td>SCLC lines</td>
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<td>Live cell target AC lines</td>
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<td>NCI-125</td>
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<tr>
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<td>NCI-69</td>
<td>166</td>
<td>1.2</td>
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Live cell RIA data are shown in Table 1 as both total cpm and binding ratios. To confirm that the antibody was binding to a cell surface component, we tested both live and fixed human lung cancer cell lines using immunofluorescences. Two AC lines, A549 and SKLU (data not shown), revealed cell surface binding where the remaining cell lines tested were all negative. Fluorescent activated cell sorter analysis was used to further demonstrate cell surface binding and to determine if all of the cells were expressing the antigen. As shown in Chart 2, only a subpopulation of cells binds the antibody.

Immunoperoxidase Staining of Nude Mouse Xenografts.

Xenograph tumor tissue of the human lung cancer cell line A549
was positive (Fig. 1). Staining was both cytoplasmic and cell surface. While xenograft tumor tissue of human lung cancer cell line NCI-H60 did not stain, similar results were found with other small cell and adenocarcinoma cell line xenografts. Biochemical Characterization of the Determinant Detected by MCA 44-3A6. Immunoprecipitation of intrinsically radiolabeled cell lysates was unsuccessful. Western blot analysis did, however, reveal a component having an estimated molecular weight of 40,000 (Fig. 2). Control blots with a 2-fold increase of antigen, no primary antibody, or an irrelevant IgG1 antibody showed no nonspecific binding (data not shown). With the antigen being preserved in both formalin fixation:paraffin embedding and after denaturation by sodium dodecyl sulfate:polyacrylamide gel electrophoresis, we wished to determine the nature of the antigen epitope, i.e., was the epitope on the antigen composed of carbohydrate or protein. A549 target plates were treated with a variety of enzyme preparations, and Table 2 shows both the total cpm and binding ratios for treated and untreated enzyme target plates. These data indicate that the epitope is sensitive to a wide variety of proteases. Periodic acid, a reagent that cleaves carbon bonds between adjacent hydroxyl groups of ringed sugars, did not significantly affect the binding of MCA 44-3A6 to a positive human lung AC cell line. These data, given the caveat of possible contaminating enzyme activity in the solutions used to treat the target plates, suggest that the epitope is proteinaceous in nature and not carbohydrate.

Detection of Shed Antigen and Serum Antigens. Many tumor cells shed components into the media of cultures or into the blood stream of tumor-bearing animals. As shown in Table 3, both 3-day-old concentrated culture supernatants and serum samples from both normal (total, 17) and lung cancer patients (total, 81) were undetectable. Two assays were designed to test either directly or indirectly the presence of the antigen. The other assay addresses the question of whether or not there are serum factors, which may or may not be the antigen, which interfere or cross-react with this MCA. Binding of this MCA to CEA was found to be negative.

DISCUSSION

This paper describes the production of a MCA directed against a novel antigen present in a subset of human lung carcinomas (see companion paper) (12). Produced against a human adenocarcinoma of the lung cell line, A549, the MCA appears to recognize a cell surface protein antigen with an apparent molecular weight of 40,000. The antigen is preserved in tissues that have been formalin fixed and paraffin embedded. It is not shed into culture supernatant nor detected in serum specimens. FACS analysis of positive cell lines suggests heterogeneity of antigen expression or, perhaps because the cells grew asynchronously, by a cell cycle-specific expression.

Several investigators have attempted to produce MCAs that will help distinguish among the various types of lung carcinomas. Brenner et al. described the production of monoclonal antibodies directed against glycoprotein antigens with molecular weights of 25,000 and 11,500 that had been purified from a squamous cell lung cancer extract by anti-β₂-microglobulin affinity chromatography (13). Varki et al. discussed three monoclonal antibodies raised against UCLA P₃, an adenocarcinoma of the lung cell line: KS 1/4 and KS 1/17, recognizing different epitopes of glycoprotein with a molecular weight of 40,000; and KS 1/9, recognizing a presumed glycolipid (14). With these antibodies, cross-reactivity was seen with all types of lung carcinomas. Masauric et al. produced four monoclonal reagents against a variety of human lung carcinoma cell lines whose provenance was not clearly detailed (15). The antibodies reacted with glycoproteins with a molecular weight of 37,000 and 19,000; 127,000; 126,000; 149,000; and 119,000. A limited number of tumor cell lines were tested to establish their binding profile. Brown et al. (16) isolated two monoclonal antibodies recognizing antigens present in squamous cell lung carcinoma. The antigens were quantitatively abundant in squamous cell carcinomas but were not restricted to this tumor type. Identification of the target antigens was not accomplished. Recently, Mulshine et al. (17) have reported two monoclonal antibodies directed against different epitopes of a M, 31,000 protein present in the cytoplasm of large cell lung carcinoma. These antibodies bound to 11 of 13 non-SCLC cell lines and 0 of 11 SCLC cell lines. The antigen was present in several cell types, which may or may not be the antigen, which interfere or cross-react with this MCA. Binding of this MCA to CEA was found to be negative.
non-lung cancer tumors. In addition, we have produced a panel of rat IgM monoclonal antibodies that recognize glycolipid antigens associated with several types of pulmonary carcinomas (4).

MCA 44-3A6 recognizes a previously undescribed antigen. Immunohistochemical staining of a large panel of human lung carcinoma specimens suggests that expression is limited to tumors demonstrating variable, including subtle, degrees of "glandular" differentiation (see companion paper) (12). The unique reactivity pattern of this MCA and the preservation of its target antigen in formalin-fixed, paraffin-embedded tissue suggest that this reagent will prove useful for the study of human lung carcinomas.

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REFERENCES

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